APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4651–4656 Vol. 63, No. 12
0099-2240/97/$04.00+0

Synthesis of Optically Active Amino Acids from α-Keto Acids with *Escherichia coli* Cells Expressing Heterologous Genes

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Received 20 June 1997/Accepted 19 September 1997

We describe a simple method for enzymatic synthesis of L and D amino acids from α-keto acids with *Escherichia coli* cells which express heterologous genes. L-amino acids were produced with thermostable L-amino acid dehydrogenase and formate dehydrogenase (FDH) from α-keto acids and ammonium formate with only an intracellular pool of NAD^+ for the regeneration of NADH. We constructed plasmids containing, in addition to the FDH gene, the genes for amino acid dehydrogenases, including i.e., leucine dehydrogenase, alanine dehydrogenase, and phenylalanine dehydrogenase. L-Leucine, L-valine, L-norvaline, L-methionine, L-phenylalanine, and L-tyrosine were synthesized with the recombinant *E. coli* cells with high chemical yields (>80%) and high optical yields (up to 100% enantiomeric excess). Stereospecific conversion of various α-keto acids to D amino acids was also examined with recombinant *E. coli* cells containing a plasmid coding for the four heterologous genes of the thermostable enzymes D-amino acid aminotransferase, alanine racemase, L-alanine dehydrogenase, and FDH. Optically pure D enantiomers of glutamate and leucine were obtained.

The worldwide market value of amino acids is approximately 2 billion dollars annually (22), and the synthesis of optically active amino acids has been extensively studied. Microbial processes have been developed for large-scale industrial production of natural proteinogenic L amino acids by fermentation. However, several other methods have been developed for the production of unnatural D and L amino acids; these methods include the enzymatic resolution of racemic amino acid amides with D- or L-aminopeptidase (13).

A method for enzymatic synthesis of L amino acids from α-keto acids with NAD(P)-dependent amino acid dehydrogenases has been proposed (10, 19). However, the application of this method to industrial production of L amino acids has been hampered by the cost of coenzymes. A multienzyme reaction system for simultaneous coenzyme regeneration (Fig. 1) has been proposed to overcome this problem. L-Leucine has been produced from α-ketoisocaproate (2-oxo-4-methylpentanoic acid) with leucine dehydrogenase (LeuDH) from *Bacillus sphaericus*, formate dehydrogenase (FDH) from *Candida boidinii*, and NADH covalently bound to water-soluble polyethyleneglycol in a reactor with an ultrafiltration membrane (6, 28). The system is also applicable to the production of several other aliphatic L amino acids, such as L-valine, L-*tert*-leucine, and L-[^{15}N]leucine. The process has been successfully scaled up for industrial production of these L amino acids (10). A similar system has been developed with phenylalanine dehydrogenase (PheDH) and FDH for the production of L-phenylalanine (1, 2). The synthesis of L-β-chloroalanine by using L-alanine dehydrogenase (AlaDH), LeuDH, and PheDH in combination with FDH has also been studied (14). However, the instability of the amino acid dehydrogenases used in these processes has hampered the efficient operation of the systems. Improvement has been achieved through the use of thermostable enzymes (18).

The only commercially available preparation of FDH is the enzyme from *C. boidinii* and is not sufficiently stable. Recently, we cloned and expressed in *Escherichia coli* the genes for bacterial FDH, which is more stable than the yeast enzyme (8). Thermostable alanine racemase (AlaR) (25), AlaDH (15), and D-amino acid aminotransferase (DAAT) (EC 2.6.1.21) (26) have been developed, and an efficient system for conversion of α-keto acids to D amino acids by the coupling of these four enzymes has been described (9) (Fig. 1). This method is also applicable to the synthesis of ^{13}N-labelled D amino acids, which are expected to be valuable in the study of mammalian neural systems (7).

The industrial use of the systems mentioned above depends chiefly on the cost of the enzymes, although intact cells of microorganisms containing the enzymes can be used as catalysts in order to decrease costs (21). In most cases, however, additional genetic improvements through metabolic engineering are required, and new functional combinations are made by the rational transfer of pathways from one organism to another (4). The transfer of the ethanol pathway from *Zymomonas mobilis* to other enteric bacteria represents an example of this approach (12). In our system, various L and D amino acids can be produced from α-keto acids if two or four functional genes are introduced into one microorganism according to the schemes presented in Fig. 1. The simultaneous expression of all enzymes in a single cell should provide additional benefit for industrial applications; the intracellular pool of NAD^+ (supplied by the cell itself) could be used for NADH regeneration without any additional supplies. We describe here a simple method for the conversion of α-keto acids to L and D amino acids with recombinant *E. coli* cells which contain plasmids with heterologous genes necessary for biotransformations.

MATERIALS AND METHODS

Plasmids and other materials. Restriction enzymes and other DNA-modifying enzymes were purchased from Toyobo or Takara and were used according to the manufacturers' instructions. Lactate dehydrogenase from rabbit muscle was obtained from Sigma. *Bacillus stearothermophilus* AlaDH was obtained from Unitika, Osaka, Japan. NAD^+ and NADH were obtained from Serva, and α-keto acids were obtained from Sigma. All other chemicals were obtained from Nacalai Tesque or Wako Pure Chemicals (Japan). The oligonucleotides were purchased

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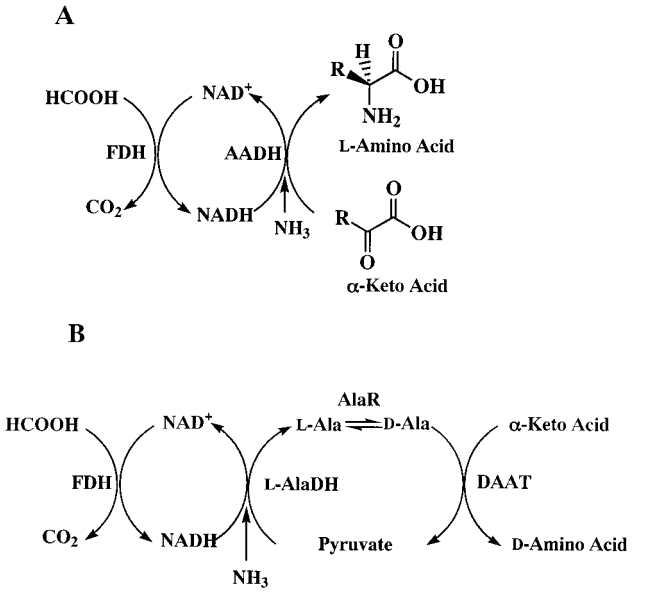

FIG. 1. Synthesis of L amino acids (A) and D amino acids (B) by coupling of enzyme reactions. AADH, L-amino acid dehydrogenase (i.e., AlaDH).

from Biologica, Nagoya, Japan. Vector plasmids pUC19 and pUC119 were used for gene cloning and expression.

DNA techniques. DNA sequencing was performed with an Applied Biosystems model 370A DNA sequencer and a dye-labelled terminator sequencing kit (Applied Biosystems). DNA synthesis by PCR was performed with a DNA Thermal Cycler (Perkin-Elmer) in 0.1 ml of a mixture containing each deoxynucleoside triphosphate at a concentration of 0.1 mM, 100 pmol of primers, 200 ng of template DNA, 0.01 ml of 10× reaction buffer (Takara), and 2.5 U of ExTaq DNA polymerase (Takara). The PCR program consisted of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min for a total of 20 cycles. Site-directed mutagenesis was carried out with a DNA mutagenesis kit from Amersham by using the protocol given by the supplier. All of the primers used are shown in Table 1.

Construction of pFDHLeuDH2, pFDHAlaDH, pFDHPheDH, and pFADA. Plasmids for the simultaneous expression of FDH and LeuDH (pFDHLeuDH2), FDH and AlaDH (pFDHAlaDH), FDH and PheDH (pFDHPheDH), and FDH, AlaDH, DAAT, and AlaR (pFADA) were constructed as shown in Fig. 2. Plasmids pULDH2, pICD301, pKPDH2, pMcFDH, pICT113p, and pMDalr3 encoding the genes for LeuDH (20), AlaDH (15), PheDH (24), FDH (8), DAAT (26), and AlaR (11), respectively, were prepared as described previously and used as template DNAs for PCR (Table 1). A unique *Sph*I site was introduced into the FDH gene in pMcFDH by site-directed mutagenesis by using the oligonucleotide shown in Table 1. The LeuDH gene was cloned by PCR by using pULDH2 as a template and was introduced into pMcFDH at the *Sph*I site to yield pFDHLeuDH1. Then, a 2.2-kb *Sal*I-*Eco*RI fragment including the structural genes for FDH and LeuDH derived from pFDHLeuDH1 was ligated with a 3.1-kb *Sal*I-*Eco*RI fragment derived from pFDH4 (27) to produce pFDHLeuDH2. pFDHAlaDH and pFDHPheDH were constructed in the same manner (Fig. 2). The *Bgl*II-*Eco*RI fragment containing the DAAT gene was isolated from pICT113p and introduced into pFDHAlaDH to obtain pFAD. Then, the *Nsi*I-*Eco*RI fragment encoding the structural gene for AlaR, which was produced with pMDalr3 by PCR, was introduced into pFAD to produce plasmid pFADA (Fig. 2).

Cultivation of recombinant *E. coli* strains and enzyme assays. Plasmid pFDHLeuDH2, pFDHAlaDH, pFDHPheDH, or pFADA was introduced into *E. coli* TG1 [F′*traD36 proAB lacI*^{q} Δ*lacZ M15* Δ(*lac-pro*) *thi hsdR ara*], and the resulting transformant was cultivated in Luria-Bertani medium (16) supplemented with 50 mg of ampicillin per liter and 0.2 mM isopropyl-β-D-thiogalactopyranoside. The cells were collected at the beginning of the stationary phase of the culture, and cell extracts were prepared by sonication. FDH, AlaDH, PheDH, and LeuDH were assayed with a Shimadzu model MPS2000 spectrophotometer by following the reduction of NAD^+ at 340 nm at 37°C by using the methods described previously (15, 20, 24, 27). DAAT was also assayed spectrophotometrically in the coupled reaction with lactate dehydrogenase (17). AlaR activity was measured in the direction from D- to L-alanine by monitoring the production of L-alanine with AlaDH (11). One unit of enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product (NADH or NAD^+) in 1 min.

Production of L and D amino acids. The clone cells were cultured as described above and washed with 0.85% NaCl. The washed cells (wet weight, 0.1 g) were suspended in 2 to 5 ml of 0.5 M ammonium formate (pH 7.5) containing 0.05 to 0.6 M α-keto acids. The standard reaction mixture for D amino acid synthesis contained 0.05 to 0.3 M α-keto acid, 0.5 M ammonium formate (pH 7.5), 5 to 10 mM pyruvate, and washed cells (wet weight, 0.2 g) in a total volume of 2 ml, and the reactions were performed at 37°C with reciprocal shaking. Aliquots of the reaction mixture were removed for analysis of substrates and products.

Identification of reaction products and determination of optical purity. The products obtained by reductive amination of α-ketoisocaproate, α-ketoisovalerate, α-ketovalerate, α-keto-γ-thiomethylbutyrate, pyruvate, phenylpyruvate, *p*-hydroxyphenylpyruvate, and α-ketoglutarate with recombinant *E. coli* cells were identified with an amino acid analyzer (model L-8500; Hitachi, Tokyo, Japan) as leucine, valine, norvaline, methionine, alanine, phenylalanine, tyrosine, and glutamate, respectively. The optical purity (the enantiomeric excess) of the amino acids produced was determined at 25°C with a high-performance liquid chromatography (HPLC) column (MCI GEL CRS 10W; Mitsubishi, Tokyo, Japan). The solvent used was 0.2 to 2.0 mM $CuSO_4$ at a flow rate of 0.5 to 1 ml/min.

The consumption of α-keto acids was monitored by HPLC with an Ultron Ps80H column (Shinwa Kako, Kyoto, Japan) at 60°C with 0.02 M perchloric acid (pH 2.0) at a flow rate of 0.8 ml/min.

RESULTS

Construction of plasmids for the production of L and D amino acids. Plasmid pFDHLeuDH2, used for simultaneous expression of FDH and LeuDH, was constructed in two steps, as shown in Fig. 2. Both LeuDH and FDH were efficiently expressed through the tandem *lac-tac* promoter of pFDHLeu

TABLE 1. Primers and template DNAs used for PCR amplification of the genes used for production of L and D amino acids

Gene	Primer	Template	Reference
FDH	5′-GCCCTGGGAATTCGAGCATGCTCAGACC-3′[a]	pMcFDH	11
LeuDH	Forward 5′-GGGCATGCGATTGAGGAGGAATGAAAG-3′[a]	pULDH2	21
	Reverse 5′-GCAGAATTCCATCCCTTATTTGTTGTT-3′[b]		
AlaDH	Forward 5′-GAAGCGGGCATGCAAAAGGAGGAAATG-3′[a]	pICD301	13
	Reverse 5′-GGCGGCTCCGAATTCAAGATCTCATCCGTGC-3′[c]		
PheDH	Forward 5′-AGCGGGCATGCTTGTTGGAGGAAGCGAAG-3′[a]	pKPDH2	22
	Reverse 5′-CATCAGTTTCATGAATTCTTTTTACCTCC-3′[b]		
DAAT	Forward 5′-TTAGCAGCGAAGATCTGAAGGATGTGGG-3′[d]	pICT113p	14
	Reverse 5′-AGCAAAACCAAAGAATTCTCACTGCAGATTATA-3′[e]		
AlaR	Forward 5′-GCGCTTTCAAGTATGCATAACAGGAAAGGC-3′[f]	pMDalr3	12
	Reverse 5′CCTTTGTCTTTTGAATTCGATTATGCACTGC-3′[b]		

[a] The underlined site is the *Sph*I restriction site.
[b] The underlined site is the *Eco*RI restriction site.
[c] The underlined sites are the *Eco*RI and *Bgl*II restriction sites (in that order).
[d] The underlined site is the *Bgl*II restriction site.
[e] The underlined sites are the *Eco*RI and *Pst*I restriction sites (in that order).
[f] The underlined site is the *Nsi*I restriction site.

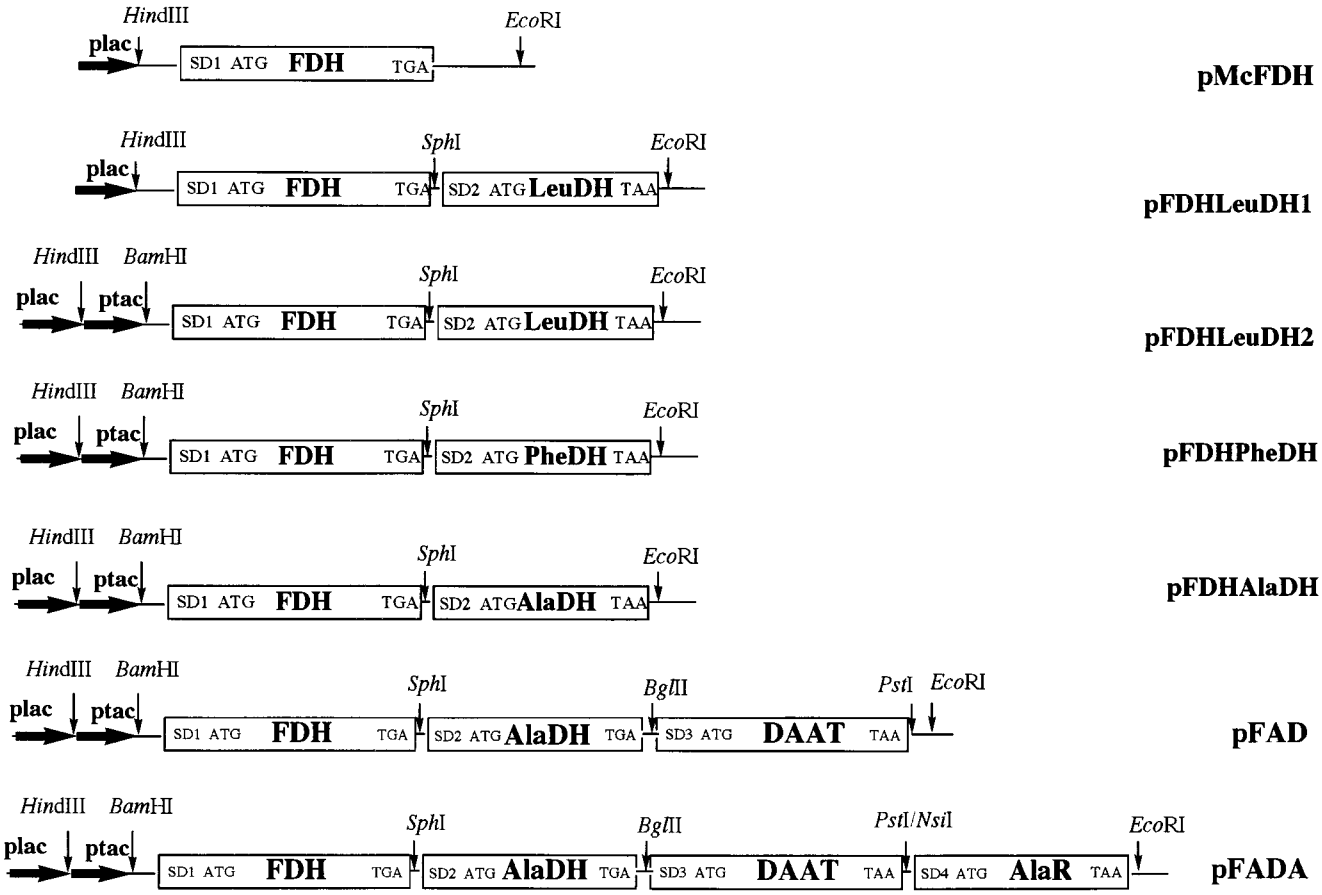

FIG. 2. Construction of the plasmids used for production of L and D amino acids by expression in *E. coli* cells.

DH2 in *E. coli* TG1. The specific activities in the clone cell extract were 1.1 U of FDH per mg and 8.2 U of LeuDH per mg. By comparing the specific activities of the homogeneous preparations of LeuDH and FDH with the specific activities in the extract, the amounts of FDH and LeuDH produced in the clone cells were each estimated to be about 7% of the total soluble protein. The levels of expression (i.e., specific activities in the cell extract) of AlaDH and PheDH were similar to those of LeuDH (1.2 U/mg for FDH and 7.3 U/mg for AlaDH with pFDHAlaDH and 1.0 U/mg for FDH and 6.7 U/mg for PheDH with pFDHPheDH). Plasmid pFADA, used for the simultaneous expression of all four genes required for the synthesis of D amino acids, was also constructed. The levels of expression of the four enzymes produced with pFADA were a little lower than the levels of expression of the enzymes described above (0.65 U of FDH per mg, 6.0 U of AlaDH per mg, 5.3 U of DAAT per mg, and 5.0 U of AlaR per mg).

Synthesis of L amino acids. LeuDH acts on various branched- and straight-chain amino acids and their α-keto analogs, and the relative rates of synthesis of L amino acids with resting cells of *E. coli* TG1 carrying pFDHLeuDH2 were similar to the rates of synthesis obtained with purified LeuDH from *Thermoactinomyces intermedius* (20) (data not shown). L amino acids with high optical purity were synthesized from α-keto acids by using ammonium formate (Table 2). L-Leucine produced from α-ketoisocaproate was crystallized in the reaction mixture during the incubation period (Fig. 3A). Crystals of L-norleucine also appeared when the concentration of this compound reached 0.14 M. L-Valine and L-norvaline were synthesized at a final concentration of 0.38 M, while the final concentrations of L-methionine and L-α-aminobutyrate were approximately 0.35 M.

L-Alanine was produced from pyruvate with *E. coli* TG1 which contained pFDHAlaDH (Table 2). The amount of L-alanine produced increased as the concentration of pyruvate increased (Fig. 3B). However, the overall yield of L-alanine decreased as the pyruvate concentration increased. Moreover, the optical purity of L-alanine decreased with prolonged incubation; the enantiomeric excess of L-alanine was 88% after 3 h, while it was only 80% after 10 h. This was probably due to the action of the AlaR produced by the host cells.

L-Phenylalanine and L-tyrosine were synthesized with *E. coli* TG1 which contained pFDHPheDH (Table 2). Because PheDH suffers from substrate inhibition at high concentrations of phenylpyruvate, the α-keto acid was added to the reaction mixture stepwise in several portions to keep its concentration no higher than 50 mM. Thus, the final concentration of L-phenylalanine was 0.3 M (50 g/liter) (Fig. 3C). L-Tyrosine was also synthesized from *p*-hydroxyphenylpyruvate in a similar manner, with a yield of 92%. L-Tyrosine crystallized in the incubation mixture due to its low solubility as the reaction proceeded. The optical purity of L-phenylalanine and L-tyrosine was 100%.

Synthesis of D amino acids. D enantiomers of glutamate and leucine were produced at high optical purities and high conversion rates with *E. coli* TG1 carrying pFADA (Table 2). The final concentration of D-glutamate produced was around 0.3 M

TABLE 2. Synthesis of L and D amino acids from α-keto acids with *E. coli* cells carrying pFDHLeuDH2, pFDHAlaDH, pFDHPheDH, or pFADA

Plasmid	Substrate	Product	Yield (%)[a]	Enantiomeric excess (%)[b]
pFDHLeuDH2	α-Ketoisocaproate	L-Leucine	97	100
	α-Ketocaproate	L-Norleucine	95	100
	α-Ketoisovalerate	L-Valine	95	100
	α-Ketovalerate	L-Norvaline	95	100
	α-Ketobutyrate	L-α-Aminobutyrate	88	100
	α-Keto-γ-thiomethylbutyrate	L-Methionine	88	100
pFDHAlaDH	Pyruvate (0.2 M)	L-Alanine	95	80
	Pyruvate (0.4 M)	L-Alanine	92	80
	Pyruvate (0.6 M)	L-Alanine	75	80
pFDHPheDH	Phenylpyruvate	L-Phenylalanine	95	100
	p-Hydroxyphenylpyruvate	L-Tyrosine	92	100
pFADA	α-Ketoglutarate	D-Glutamate	85	100
	α-Ketoisocaproate	D-Leucine	76	100
	α-Ketocaproate	D-Norleucine	70	88
	α-Keto-γ-thiomethylbutyrate	D-Methionine	80	90
	α-Ketoisovalerate	D-Valine	85	92
	α-Ketovalerate	D-Norvaline	90	35
	α-Ketobutyrate	α-Aminobutyrate	95	0
	Phenylpyruvate	D-Phenylalanine	15	ND[c]
	p-Hydroxyphenylpyruvate	D-Tyrosine	5	ND

[a] The yields were determined after 12 h of incubation.
[b] The optical purity was determined by HPLC.
[c] ND, not determined.

(Fig. 3D). Norvaline, valine, and α-aminobutyrate were also produced with high yields. However, α-aminobutyrate was synthesized as a racemic mixture because it is racemized by AlaR. D-Norvaline was obtained at an enantiomeric excess of only 35%. This compound was also racemized by the racemase, although at an extremely low rate. D-Valine, D-methionine, and D-norleucine also suffered from contamination by the antipodes at concentrations of 4, 5, and 6%, respectively. This was

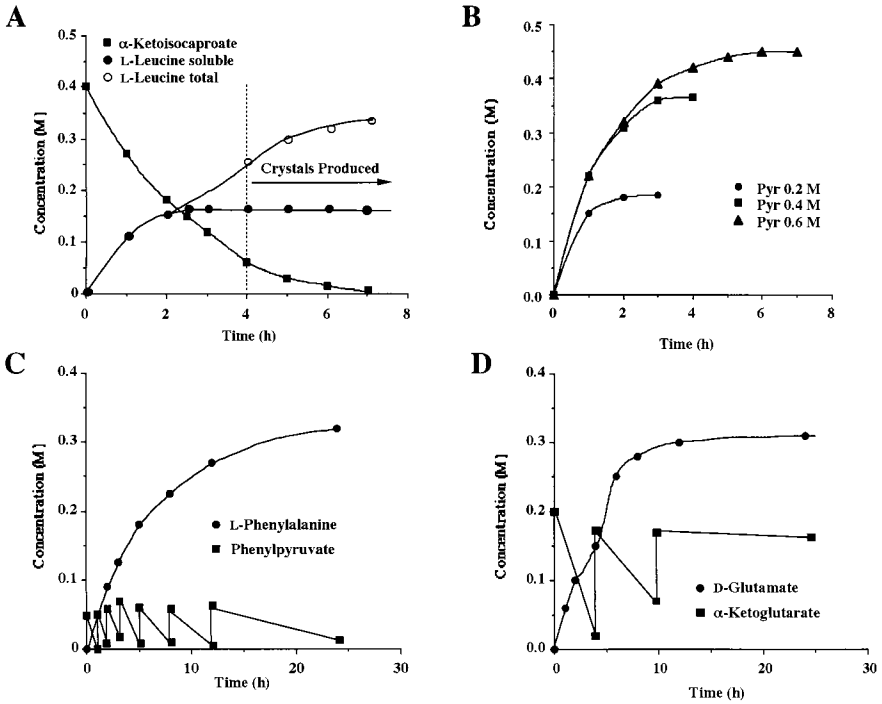

FIG. 3. Time course for the production of L-leucine (A), alanine (B), L-phenylalanine (C), and D-glutamate (D) with *E. coli* cells carrying pFDHLeuDH2 (A), pFDHAlaDH (B), pFDHPheDH (C), and pFADA (D), respectively. (A) After 4 h of incubation (dotted line) crystals of L-leucine appeared in the reaction mixture. Finally, the reaction mixture was solidified with the crystals. (B) The L-alanine produced had an optical purity of approximately 80%. (C) Sodium phenylpyruvate was added to the reaction mixture stepwise. (D) α-Ketoglutarate was added after 4 and 10 h of incubation at a final concentration of approximately 0.2 M.

probably due to the action of AlaDH, because these amino acids are not racemized by AlaR (9). D-Phenylalanine and D-tyrosine, which are poor substrates for DAAT, were synthesized with yields of less than 15%.

DISCUSSION

We show here the usefulness of FDH, DAAT, and AlaR, as well as L-amino acid dehydrogenases, such as LeuDH and PheDH, for the production of optically active amino acids. The simultaneous expression of all enzymes in a single cell allowed the efficient recycling of NADH. We found that the intracellular concentration of NAD^+ is high enough to produce amino acids at final concentrations of approximately 0.3 M. We were not, however, able to obtain production of higher concentrations, probably due to the degradation of the coenzyme in the cells, as has been reported previously (23).

Optically pure L-phenylalanine was synthesized with a yield of 99% with a mixture of acetone-dried cells of *B. sphaericus* and *C. boidinii* as sources of PheDH and FDH, respectively (2). In our study, a similar yield was obtained by using the cells of a single strain, *E. coli* TG1 carrying pFDHPheDH, and the cells were not treated with acetone, which increased the permeability of the cell membrane for the NADH added.

We used resting cells of *E. coli* as the catalyst. However, optically active amino acids were also produced in a growing cell system by using Luria-Bertani or M9 medium (16) supplemented with ammonium formate and α-keto acid (data not shown). Our preliminary experiments showed that α-keto acids, particularly branched-chain and long-chain α-keto acids, are toxic and inhibit the growth of *E. coli* when they are added at concentrations of only 15 to 30 mM. Therefore, the resting cell system is much more convenient than the growing cell system for producing high concentrations of amino acids.

We have shown previously by using the purified-enzyme system (9) that low optical purity of the D amino acids produced is mostly due to the action of AlaDH toward α-keto acids other than pyruvate. Similar results were obtained with the cell systems discussed here. *E. coli* used as a host should have several L-amino acid aminotransferases, which may decrease the optical purity of the D amino acids produced. Although no racemase activity was found in amino acids other than alanine in *E. coli*, the optical purity of the L-alanine produced in our system decreased due to the action of the AlaR of the host cells. Thus, yeasts, which inherently lack AlaR activity, or AlaR-deficient mutant strains of *E. coli* may facilitate enantiospecific synthesis of L-alanine when they are used as the hosts for AlaDH and FDH genes. Optically pure L-β-chloroalanine was synthesized with a 90% yield from β-chloropyruvate with purified preparations of AlaDH and FDH (14). Because AlaR is efficiently inactivated by β-chloroalanine (3), our present system in which *E. coli* TG1/pFDHAlaDH cells are used probably serves as an efficient tool for the production of optically pure L-β-chloroalanine.

As exemplified by the production of leucine enantiomers with 100% optical purity as shown here, our system should be particularly suitable for the synthesis of unnatural L and D amino acids with bulky side chains, such as *tert*-leucine, which are used as building blocks for potent human immunodeficiency virus protease inhibitors, antitumor agents, etc. (5). The L enantiomer of *tert*-leucine has been produced efficiently in a membrane reactor system by using purified LeuDH and FDH, and the D enantiomer has been obtained chemically only by using many complicated steps. Our system should serve as a simple method for the production of D-*tert*-leucine.

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